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PREPARATION AND USE
OF
PURE CULTURES FOR LEGUME INOCULATION

By
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INTRODUCTION

Soil inoculation has been practiced in limited areas for some years. The very great importance of this practice, however, as a factor in increasing the fertility of the soil, as well as insuring a better crop, is just coming to be realized by practical agriculturists and other students of the soil. Here in the Northwest the matter has been receiving more and more attention, and the Washington Agricultural Experiment Station, in its endeavor to serve in greatest measure the needs of the State, has sought to make available to all the benefits to be derived from this practice.

Inoculation of fields by means of soil taken from areas already inoculated, which are free from weeds and other pests, is not always practicable, and the use of commercial cultures, as well as of those formerly supplied from the U. S. Department of Agriculture, has not always proved successful. For this reason this Station, two years ago, undertook to supply farmers of the State with fresh viable cultures in order to insure maximum results. The appreciation with which this work has been received is evidenced by the fact that during the last year pure cultures of the *Pseudomonas radicicola* have been supplied for over 9300 acres of leguminous crops.

Although the use of pure cultures supplied from this Station had given excellent results in the field, we did not feel justified in assuming that we were necessarily following the best

methods in the preparation and handling of these cultures. Consequently, measures were undertaken to learn how the work was being done at other tations, in order that we might improve upon our own practices and give to others the benefits of our investigations. On the whole, our inquiries met with prompt and hearty response from those interested in the matter throughout the country. In a few instances we were not successful in getting the information sought, but the returns seemed sufficiently full to warrant a digest of the material, and the drawing of some conclusions.

LABORATORY EQUIPMENT REQUIRED

Where the usual bacteriological laboratory apparatus is available no extra equipment is found necessary, other than supplies of bottles or other containers in which the cultures are grown and shipped.

IN WHAT FORM SUPPLIED TO THE FARMERS

In general, cultures are supplied upon nitrogen-free solid media in tubes and bottles. In some instances, liquid media are used, and in others the cultures are supplied to the farmers in sterilized sand or soil. At least one Station makes a practice of furnishing soil from inoculated fields. In our practice, during the last two years, sterilized sand in tin soil cans has been used for this purpose, the organisms being cultivated in flasks, transferred to the sand by use of sterile water, and the can then sealed just previous to shipment. This method has proved satisfactory, so far as positive inoculation is concerned, but handling the sand in cans is cumbersome, and transportation charges are heavy. In addition, some complaints have been made that the sand causes excessive wear on seeding machinery.

COST OF CULTURES

The general practice among Experiment Stations and State Departments of Agriculture is to supply cultures to the farmer at cost. This cost varies, naturally, with the different methods of preparation and handling, but reports indicate that in any

case cultures can be supplied to farmers as a cost of from 25 cents to 40 cents per acre. Some Stations supply the cultures in limited amounts free of charge. Our own practice has been to charge a uniform price of 25 cents per acre, which we have found sufficient. Increase in the price of raw materials and of labor may necessitate a slight advance in price, but such is not anticipated.

STRAINS OF PSEUDOMONAS RADICICOLA USED

For the most part, is is believed that the same strain of *Ps. radicicola* infects the roots of alfalfa and sweet clover, and therefore the same culture may be employed for both.

A second strain is effective for the clovers belonging to the genus trifolium.

A third strain isolated from the vetch is applicable for the varieties of this legume. While some recommend the use of the vetch strain for garden peas, we have felt safer in the use of a strain isolated from field peas, for this legume.

Our tests have shown that the strain isolated from the field pea may be used successfully, not only for the field and garden varieties, but also for sweet peas.

In like manner, we have been successful in the use of a strain isolated from garden beans for all varieties of this legume.

Cow peas and soy beans are not planted to a large extent in this section, so we have had no occasion to investigate the strains of the organism necessary for the inoculation of these crops. From such information as we have, each seems to require its own particular strain.

It is common practice at the various Stations to supply strains of the organism freshly isolated from locally grown legumes for which crops they are later to be used. This, of course, is the safest and best practice.

APPLICATION OF CULTURE TO SEED

Considerable variation in methods employed in the application of cultures to seed has been noted. In some instances it is recommended that glue be added to the suspension of bac-

teria in order to cause them to adhere to the seed, while others advise specifically against this practice. The cultures themselves are more or less gelatinous in nature, and special measures to cause them to adhere to the seed do not appear necessary. We can see no advantage to be derived from the use of glue. On the other hand, since glue is usually heavily infested with bacteria of various kinds, antagonism of types may result in the destruction of the desirable nitrogen-fixing forms.

Sugar, and also milk, are sometimes used in the preparation of the suspensions for seed treatment, the object being to furnish nutrients for the bacteria. We have never investigated the desirability of this practice. Uniformly good results have been obtained without the use of materials of this sort, and they do not appear to us necessary. Furthermore, addition of these materials complicates the work of the farmer, and experience has shown us that this should be avoided whenever possible.

In the application of any culture to seed, care must be taken to thoroughly and evenly moisten the seed with the suspension of the bacteria. The seed of alfalfa and clover must be allowed to dry sufficiently to facilitate planting, but the drying should be done away from direct sunlight. With larger seeds, such as peas and beans, it is unnecessary to dry them, and the most convenient practice is to add the culture to the seed in the field as it is put into the drill. All treated seed should be planted as soon as possible. Handling of all cultures and of seed after treatment should be such that the bacteria will not be killed by exposure to direct rays of the sun.

REINOCULATION

Whether reinoculation is necessary the following year, or for several years after, is a question which is very frequently asked. It is impossible to answer this question in every case as it has been shown that in some regions limiting factors, probably drought and soil acidity, are operative, which cause the death of the organisms in the soil and make reinoculation necessary, even during the following season. In other regions one inoculation has been found sufficient for a period of years.

In the Palouse country we have found the soil heavily inoculated with the nodule-forming bacteria three years after their first introduction, although two non-leguminous crops had been introduced between the leguminous crops in the rotation.

This subject of the factors which limit the retention of these organisms in the soil is one which needs further careful investigation. Likewise, the question of whether or not these organisms when existing free in the soil may perform any desirable function is worthy of further study.

LABORATORY CULTIVATION

To the laboratory worker the methods employed in the cultivation of the bacteria and their preparation for shipment are of special interest. Because of the great surface which is afforded, flat bottles or "Blakes" are commonly used for this purpose. Test tubes are used to some extent, though bottles of 2, 4, and 8 ounce capacity appear best adapted for laboratory cultivation. We have used 8 ounce "Baltimore ovals" and also one-half-pint whiskey flasks for this purpose, with satisfaction, but any flat bottle which is fairly insoluble, and will stand sterilization, will serve. For all round laboratory use the "Baltimore oval" bottle (Whitall Tatum Co., Philadelphia, Chicago and San Francisco) is unsurpassed.

The medium is sterilized in the bottle, the bottle is then laid flat on the table or shelf to allow the medium to solidify, and when cold is ready for use. We have found inoculation most easily and satisfactorily effected by introducing 1 cubic centimeter of a suspension of the organism into the flask, with a sterile pipette, and then distributing the same over the surface of the medium by tilting back and forth.

Incubation is carried on at ordinary room temperature, as a rule, either in cupboards, or in rooms provided with shelves for the purpose. Such rooms should preferably be dark, but at least the light should be subdued.

The duration of incubation preceding the sealing for shipment varies with different workers. Some report 48 hours as sufficient, but from one to two weeks is more commonly

employed. We have rarely used cultures of less than one week's growth.

The amount of culture required per acre has not been standardized, though the growth of a solid medium in a flat 2-ounce bottle is usually considered sufficient for the inoculation of 2 acres. Two weeks' growth on the surface of the medium in an 8 ounce bottle is considered sufficient for from 10 to 15 acres.

How long cultures remain viable after being sealed is a very important question. Some workers consider that cultures are not reliable after 20 days from the time of sealing; others think one month not too long. Where sand is employed as the vehicle for distribution, longer periods are permissible than when cultures are supplied to the farmer upon the original culture medium. We have found the organisms alive in our sand preparations, and capable of forming nodules, as long as 8 months after the cans were sealed. One cubic centimeter of the liquid drawn from the sand showed as high as 3,000,000 bacteria at that time. However, we do not advise the use of such cultures, and have, in fact, advised against the use of cultures over three weeks old. Our investigations on the viability of cultures in sand have not been completed.

This is a very important consideration, as failure of cultures to produce good results in field practice has doubtless been due very largely to the fact that these cultures were too old, and the organisms were either dead, or their vitality greatly weakened. Cultures, then, should be obtained directly from a nearby laboratory, if possible, and in any case the purchaser should satisfy himself that all cultures used are fresh.

INVESTIGATION OF CULTURE MEDIA*

In our survey we found but slight uniformity in the composition of the culture media used by different workers. Being anxious to determine which of these was best adapted for

*We are greatly indebted to Mr. L. T. Ruehl for his valuable assistance in the laboratory studies connected with this part of the investigation.

general cultivation of the various strains required in our work, we undertook a comparative study of both liquid and solid media made according to the formulae used by different Station workers. Since it has been our sole purpose to compare the relative merits of the different culture media, no reference is here given to the particular Station using a given formula.

Liquid Media

The different liquid media investigated were:

No. 1		No. 2	
Tap water	1000.00 cc.	Tap water	1000.00 cc.
KH_2PO_4	1.00 gm.	KH_2PO_4	1.00 gm.
$MgSO_4$	0.10 gm.	$MgSO_4$	0.10 gm.
Saccharose	10.00 gm.	Dextrose	20.00 gm.
NaCl, $FeSO_4$, $MnSO_4$	traces	NaCl, $FeSO_4$, $MnSO_4$	traces

No. 3		No. 4	
Tap water	1000.00 cc.	Tap water	1000.00 cc.
KH_2PO_4	1.00 gm.	KOl	2.00 gm.
$MgSO_4$	0.10 gm.	$MgSO_4$	1.00 gm.
Mannit	20.00 gm.	NaCl	1.00 gm.
NaCl	trace	$CaSO_4$	1.00 gm.
		$Ca_3(PO_4)_2$	1.00 gm.
		Saccharose	10.00 gm.

No. 5		No. 6	
Tap water	1000.00 cc.	Nutrient broth (Standard)	1000.00 cc.
$(NH_4)_2HPO_4$	8.00 gm.	Saccharose	50.00 gm.
$MgSO_4$	0.50 gm.		
Saccharose	8.00 gm.		

No. 7		No. 8	
Distilled water	1000.00 cc.	Tap water	1000.00 cc.
KH_2PO_4	1.00 gm.	KH_2PO_4	1.00 gm.
$MgSO_4$	0.50 gm.	$MgSO_4$	0.05 gm.
Saccharose	10.00 gm.	Dextrose	10.00 gm.

No. 9		No. 10	
Tap water	1000.00 cc.	Tap water	1000.00 cc.
$Mg_3(PO_4)_2$	0.50 gm.	K_2HPO_4	1.00 gm.
Dextrose	10.00 gm.	Saccharose	12.00 gm.
		10% solution of $CaCl_2$, $FeCl_3$, $MgSO_4$, $MnSO_4$	3 drops

No. 11		No. 12	
Tap water	1000.00 cc.	Nutrient broth (Standard)	1000.00 cc.
KH_2PO_4	1.00 gm.	Dextrose	10.00 gm.
$MgSO_4$	0.20 gm.		
Saccharose	10.00 gm.		

Technique of the Test

Culture solutions prepared according to the above formulae were distributed in quantities of 65cc. into 8-ounce flasks, and sterilized. Inoculation of these was made from cultures one week old on a medium of the following composition:

Tap water	1000.00 cc.
K_2HPO_4	1.00 gm.
Saccharose	10.00 gm.
Agar agar	20.00 gm.

A suspension was made by washing the growth from the surface of the medium with a small quantity of sterile water, and 1 cubic centimeter of this suspension was introduced into each flask, with aseptic precautions. The flasks were then well shaken and placed in lockers at room temperature to incubate. Tests were made in triplicate on each of the five strains for which we have demand, namely, those isolated from alfalfa, beans, clover, peas, and vetch. After incubation for 10 days each culture was well shaken and one standard loopful was transferred to 1 cubic centimeter of sterile water previously placed in the sterile Petri dish. The plates were then paured with nitrogen-free agar of the composition given above.

After 4 days incubation at room temperature these plates were examined for colony growth.

Upon the basis of these tests the values of the media were found to stand in the following order:

Medium	No. 5.....1st	Medium	No. 10..... 6th
"	No. 6.....2nd	"	No. 11..... 7th
"	No. 12.....3rd	"	No. 3..... 8th
"	No. 2.....4th	"	No. 1..... 9th
"	Nos. 4, 8, 9.....5th	"	No. 7.....10th

Solid Media

The different solid media investigated were:

No. 1		No. 2	
Tap water	1000.00 cc.	Tap water	1000.00 cc.
KH_2PO_4	3.00 gm.	KH_2PO_4	0.20 gm.
Saccharose	10.00 gm.	Mannit	15.00 gm.
Maltose	10.00 gm.	$MgSO_4$	0.20 gm.
Leachings from 15 gms. of hardwood ashes		$CaSO_4$	0.10 gm.
		$CaCO_3$	0.50 gm.
		NaCl	0.20 gm.
Agar agar	15.00 gm.	Agar agar	15.00 gm.

No. 3		No. 4	
Tap water	1000.00 cc.	Distilled water	1000.00 cc.
KH_2PO_4	1.00 gm.	KH_2PO_4	1.00 gm.
$MgSO_4$	0.20 gm.	$MgSO_4$	0.20 gm.
Maltose	10.00 gm.	Saccharose	10.00 gm.
Agar agar	10.00 gm.	Agar agar	10-15.00 gm.

No. 5		No. 6	
Tap water	1000.00 cc.	Tap water	1000.00 cc.
Saccharose	10-15.00 gm.	K_2HPO_4	1.00 gm.
Leachings from 5 gms. of hardwood ashes		Saccharose	10.00 gm.
		Agar agar	20.00 gm.
Agar agar	10.00 gm.		

No. 7	
Tap water	1000.00 cc.
$(NH_4)_2HPO_4$	8.00 gm.
$MgSO_4$	0.50 gm.
Saccharose	8.00 gm.
Agar agar	15.00 gm.

No. 8	
Tap water	1000.00 cc.
K_2HPO_4	0.20 gm.
$MgSO_4$	0.20 gm.
$CaSO_4$	0.10 gm.
$CaCO_3$	5.00 gm.
NaCl	0.20 gm.
Saccharose	15.00 gm.
Agar agar	12-15.00 gm.

A small quantity of $CaCO_3$ was also added to each flask.

The technique of the tests upon these media was essentially the same as that of the culture solutions,—the source of cultures, the temperature, periods of incubation, etc., being practically identical. In determining the amount of growth, however, the plates were prepared by first making a setrile water suspension of the growth in each flask, equal in volume to that of the liquid media, and then proceeding as in the case of the liquid cultures. In this way it was possible to compare directly the values of the solid and the liquid media.

Of the eight solid media tested, four (Nos. 2, 4, 6, and 8) were so far superior to the others that these latter were discarded. These four were so nearly equal in the amount of growth that a choice among them could not be made. Comparison of the plates from these with the plates from the liquid media showed the amount of growth present to be many times that obtained in the best of the liquid cultures.

In order to determine, if possible, which of these four media was best suited for use in the shipment of cultures they were subjected to further tests. All bottles were sealed with paraffin and placed in storage at room temperature. At the end of 10 days, one lot, consisting of cultures of each strain on each medium, was removed from storage and tested as before; 130 cubic centimeters of sterile water were used in making the suspensions instead of 65 cubic centimeters, as in the first test. The growth obtained upon these plates failed to show

any distinguishable differences in the value of the four solid media. However, nothwithstanding the fact that the cultures had been sealed for 10 days, even at this dilution the growth was fully the equivalent of that found on the plates of liquid medium No. 5 when that was at its best.

At the end of 20 days another lot similar to the above was taken from storage, and a like test performed. Again we found no distinguishable advantage of one medium over the others as shown in the amount of growth produced. There was a marked reduction in the numbers of viable organisms, however, the growth in this instance being approximately one-half as great as that obtained upon the plates made ten days before. It will be seen, then that these cultures after being sealed for 20 days contained as many viable organisms as the best of the liquid cultures having free access of air.

In view of the fact that liquid cultures are difficult to ship satisfactorily, and also are much inferior to the solid media in production of growth, we feel that any one of the four solid media considered above should be used in preference to any of the liquid media tested for the cultivation and shipment of cultures.

We have not made extended tests of media Nos. 2, 4, and 8, but we have used medium No. 6 in the cultivation of the *Pseudomonas radicicola* in our laboratories for the last two years, with excellent results. It is simple in formula, and easy to prepare.

CONCLUSION

In brief, the facts brought out by this survey may be summarized as follows:

1. Cultures may be furnished to farmers with but little addition to the ordinary bacteriological laboratory equipment.

2. Cultures supplied to the farmer at cost need not exceed the price of 40 cents per acre.

3. Strains of the *Pseudomonas radicicola* used are best obtained by direct isolation from locally grown legumes of the variety for which they later are to be used.

4. In applying cultures to the seed the use of glue or other adhesives is not necessary, and the use of nutrients in the preparation of the bacterial suspension is of questionable value.

5. Solid media are superior to liquid media for laboratory cultivation and shipment of pure cultures of the *Pseudomonas radicicola*.